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# Effects of short chain alkanols on the inducible nitric oxide synthase in a glial cell line

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**1** Ethanol inhibits inducible nitric oxide synthase (iNOS) expression in C6 glioma cells by an unknown mechanism. Because relatively high concentrations are needed for inhibition in drug-naïve cells ($IC_{50} \approx 150$ mM), suppression due to cytotoxicity is one possible mechanism that has not been ruled out. Therefore, the present study examined the effects of ethanol and other alkanols on C6 glioma cell viability and iNOS activity to better understand the mechanism for inhibition.

**2** iNOS expression was induced in cell culture with lipopolysaccharide and phorbol ester treatment. Nitrite accumulation in culture medium, the *in vitro* conversion of [$^3$H]-L-arginine to [$^3$H]-L-citrulline, and immunoblotting were used to quantify iNOS induction and activity. Trypan blue exclusion, extracellular release of lactate dehydrogenase, and quantity of total cell protein were used as measures of viability.

**3** Short chain alkanols, methanol through 1-heptanol, concentration-dependently inhibited nitrite accumulation. Longer chain alkanols, 1-octanol and 1-decanol, did not except at cytotoxic concentrations. Experiments indicated short chain alkanol inhibition was not due to direct actions on iNOS catalytic activity, but that it transpires during iNOS induction. Immunoblots showed reduced iNOS protein levels.

**4** Correlation analysis ruled out iNOS inhibition as being due to decreased cell number, total cell protein, or cell viability. In contrast, there was significant correlation with physical measures of lipophilicity.

**5** In conclusion, inhibition of iNOS expression by ethanol and other short chain alkanols is not due to cytotoxicity. Instead, the strong correlation with lipophilicity suggests the inhibition derives from an interaction with unknown hydrophobic cellular sites.

**Keywords:** iNOS; nitric oxide; ethanol; short chain alkanols; C6 glioma cells; cytotoxicity; gene expression

**Abbreviations:** ANOVA, analysis of variance; CI, confidence interval; C-1, methanol; C-2, ethanol; C-3, 1-propanol; C-5, 1-pentanol; C-7, 1-heptanol; C-8, 1-octanol; C-10, 1-decanol; FBS, foetal bovine serum; HBSS, Hank's balanced salt solution; iNOS, inducible nitric oxide synthase (*Nos*2 gene product); LDH, lactate acid dehydrogenase; LPS, lipopolysaccharide; PBST, phosphate buffered saline containing 0.05% Tween 20; PMA, phorbol 12-myristate 13-acetate

## Introduction

Incubation with ethanol reduces the expression of the inducible isoform of nitric oxide synthase (iNOS; L-arginine, NADPH:oxygen oxidoreductase, nitric oxide forming; EC 1.14.13.39) in C6 glioma cells (Militante *et al*., 1997). Reduction of nitrite accumulation in the culture medium, a marker for nitric oxide production by iNOS, is time and concentration-dependent (Syapin, 1995). Studies using *in vivo* ethanol administration also observed inhibition of iNOS expression (Spolarics *et al*., 1993; Xie *et al*., 1995), indicating this effect of ethanol is not cell type specific (see however, Durante *et al*., 1995; Naassila *et al*., 1996). Recent studies using other drugs of abuse indicate the suppression of glial iNOS by ethanol appears unique (Syapin, 1997).

The structure-activity relationship is a classical tool to provide insight into the specificity and spatial requirements for pharmacological effects by a series of related compounds. Previous studies of alkanols (*n*-alcohols) and related compounds on ligand-gated ion channels (Wood *et al*., 1991; Machu & Harris, 1994; Li *et al*., 1994) and firefly luciferase (Franks & Lieb, 1985) observed correlations between alkanol potency and carbon chain length, with a loss of activity (cutoff phenomena) occurring for either shorter (Wood *et al*., 1991) or longer chain alkanols (Machu & Harris, 1994; Li *et al*., 1994; Franks & Lieb, 1985). These results (Wood *et al*., 1991; Machu & Harris, 1994; Li *et al*., 1994; Franks & Lieb, 1985) were interpreted to show that alkanols and related compounds interact with spatially-defined hydrophobic sites on proteins to have effects independent of their lipid disordering properties. Identification of so-called 'alcohol-sensitive' proteins is important for a better understanding of the acute and chronic effects of ethanol.

In the present study, the structure-activity relationship for suppression of iNOS expression by ethanol and other short chain alkanols was examined in C6 glioma cells. Additional studies were performed to better understand the mechanism underlying the alkanol suppression. These included possible direct interactions with iNOS catalytic activity and effects on cell viability. A correlational analysis was used to assess activities that contribute to iNOS suppression by short chain alkanols.

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## Methods

### *Cell culture*

Stock and experimental cultures of C6 cells were prepared and maintained as previously described (Syapin, 1995) and used to study glial iNOS induction. Briefly, cell stocks were grown in high glucose-containing Dulbecco's modified Eagle's medium with 5% foetal bovine serum (FBS) and experimental cultures were grown in medium with 2.5% FBS. All cultures were maintained at 37°C inside humidified incubators with 5% $CO_2$ 95% air. The culture medium was replenished 2–3 days after seeding and every other day thereafter.

### *Drug treatment of cells*

Treatment of cell cultures with alkanols was performed as described previously for ethanol (Syapin, 1995; Militante *et al*., 1997). Experimental cultures were used after 5 or 7 days growth. Briefly, the growth medium was removed and the cells rinsed and replenished with serum-free Dulbecco's modified Eagle's medium containing the desired concentration of alkanol. The alkanols and their concentrations used were: methanol (0, 100, 200, 300 and 400 mM); ethanol (0, 25, 50, 100 and 200 mM); 1-propanol (0, 25, 50 and 100 mM); 1-pentanol (0, 1, 10 and 25 mM); 1-heptanol (0, 0.5, 1, 2, 4 and 5 mM); 1-octanol (0, 0.5, 1, 2 and 4 mM), and 1-decanol (0, 0.1, 0.2, 0.3, 0.4 and 0.6 mM).

For iNOS induction, phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS) were added sequentially to individual cultures (400 ng $ml^{-1}$ and 500 ng $ml^{-1}$ final concentrations, respectively) unless stated otherwise. The cultures were placed inside re-sealable plastic bags containing a 400 ml reservoir of either water or aqueous alkanol solution at a concentration equivalent to that of the culture medium, gassed with 5% $CO_2$: 95% air and sealed. Short chain alkanols were added directly to culture medium at 37°C and to room temperature water immediately prior to use. 1-Heptanol was added to serum-free medium, capped tightly and brought to 37°C for at least 30 min with periodic vortex mixing prior to use. Reservoirs containing aqueous solutions of 1-heptanol were sealed and stirred at room temperature for at least 120 min prior to use. The longer chain alkanols, 1-octanol and 1-decanol, were first prepared as methanolic solutions and then diluted to give saturated solutions (4 mM and 0.6 mM in 80 mM methanol, respectively) by constant stirring at 4°C overnight in serum-free medium or water, as described by Wood *et al*. (1991). Control cultures were exposed to 80 mM methanol alone, which had no effect on iNOS activity (data not shown, see also Figure 1). Saturated solutions in serum-free medium were diluted with cold medium to the desired concentration and warmed to 37°C prior to use. Saturated water solutions were diluted with room temperature water prior to use. Sterile plastic ware (flasks, tubes and pipettes) was used exclusively when working with longer chain alkanols to reduce losses due to adherence to glass surfaces.

In general, culture medium was collected 24 h after initiation of iNOS induction for measurement of nitrite accumulation, and in some cases for LDH activity. The cells were rinsed in Hank's balanced salt solution (HBSS), dried, and dissolved in 1 M NaOH to determine total cell protein by the BCA method (Smith *et al*., 1985). In some instances, the cells were harvested and used to prepare a cytosolic fraction or used to determine cell number and viability.

For post-induction experiments, control cultures were treated with 150 u $ml^{-1}$ of interferon-$\gamma$ and 500 ng $ml^{-1}$ of LPS to initiate iNOS induction. The medium was collected after 24 h and analysed for verification of nitrite accumulation. The cells were rinsed once and replenished with fresh, 37°C serum-free medium containing the desired concentration of alkanol. The cultures were then incubated for 24 h at 37°C in gassed, sealed plastic bags as described above. The medium was again collected and analysed for new nitrite accumulation. The cells were rinsed in HBSS, dried, dissolved in 1 M NaOH and protein content determined (Smith *et al*., 1985).

### *Determination of intact cell iNOS activity*

This procedure has been described previously (Syapin, 1995). Briefly, iNOS activity was assessed using the Griess reagent (Green *et al*., 1982) to measure nitrite accumulated in the medium of individual cultures after treatment with inducing agents (see above). Measurements were performed in duplicate. Sodium nitrite was added to control medium and used to prepare a standard curve for quantitation.

### *Determination of* in vitro *iNOS activity*

A pooled cytosol fraction was prepared from control cultures after 24 h treatment with 400 ng $ml^{-1}$ of PMA plus 500 ng $ml^{-1}$ of LPS using published methods (Galea *et al*., 1992), with minor modifications (Militante *et al*., 1997). Culture medium was collected from each of three or five induced cultures (100 mm diameter dishes), pooled and later analysed for verification of nitrite accumulation. The cells were immediately washed with cold HBSS, scraped from the dishes and homogenized in 1.4–5 ml of ice-cold buffer A [50 mM Tris-HCl (*p*H 7.8), 320 mM sucrose, 1 mM EDTA, 1 mM DL-dithiothreitol, 0.2 mM benzamidine, 10 $\mu$M pepstatin A, 10 $\mu$g $ml^{-1}$ chymostatin, 10 $\mu$g $ml^{-1}$ phenylmethylsulphonyl fluoride]. The homogenate was centrifuged at 100,000 × *g* for 30 min and the supernatant collected and dialyzed for 2 h against cold buffer B [50 mM Tris-HCl (*p*H 7.8), 1 mM EDTA, 1 mM DL-dithiothreitol, and 0.2 mM benzamidine] to remove low-molecular weight solutes.

The dialyzed cytosolic extract was stored in aliquots at −80°C until assayed for *in vitro* iNOS activity by following the conversion of [$^3$H]-L-arginine to [$^3$H]-L citrulline, as described (Feinstein *et al*., 1994). Briefly, 50 $\mu$l of the cytosol (98–148 $\mu$g protein) was incubated at 37°C for 30 min with 50 $\mu$l of buffer C [Tris-HCl (*p*H 7.8 100 mM), NADPH 1 mM, FAD 10 $\mu$M, tetrahydrobiopterin 10 $\mu$M, L-arginine 10 $\mu$M and 1.0 $\mu$Ci $ml^{-1}$ [$^3$H]-L-arginine] containing a 2× concentration of a specific alkanol. No alkanol was added to buffer C for control samples. 1-Heptanol and 1-octanol were added to buffer C either directly or as methanolic solutions (methanol 80 mM and 1-heptanol 10 mM or methanol 80 mM and 1-octanol 2 mM). The reaction was halted by the addition of cold 20 mM HEPES buffer, *p*H 5.5. Dowex-50W resin ($Na^+$-form) was added to bind free [$^3$H]-L-arginine, and the sample was centrifuged briefly. The [$^3$H]-L-citrulline recovered in the supernatant was assayed by liquid scintillation counting. Each individual assay used one batch of cytosol ($n=6$) and each data point represents a separate incubation of the extract with buffer C.

### *Trypan blue exclusion test*

Experimental cultures were exposed to the highest tested concentration of alkanols C-1 to C-7 in the presence or absence of PMA plus LPS for 24 h. Afterwards, the cells were detached by treatment with 0.25% trypsin, collected by centrifugation, resuspended in medium with 2.5% FBS and stained with 0.2%

trypan blue. The number of total and blue-stained cells was determined with a hemocytometer by duplicate counts on at least 200 cells per count.

### *Determination of LDH release*

The medium from the cultures used for the trypan blue exclusion test was collected and analysed for the presence of LDH (Wroblewski & LaDue, 1955) using conditions modified slightly from the published procedure. Background activity was determined on 200 $\mu$l of media by addition of 767 $\mu$l of NADH (0.087 mg ml$^{-1}$ in 0.1 M potassium phosphate buffer) followed immediately by 33 $\mu$l of phosphate buffer and spectroscopic measurement of the rate of change at 340 nm. Stimulated activity was measured by substituting 33 $\mu$l of a 22.7 mM pyruvate solution for phosphate buffer. Absorbance readings were recorded every 20 s for 3 min on a Shimadzu model UV-160 recording spectrophotometer. LDH activity was calculated by subtracting background activity from stimulated activity after converting the rate of decline at $A_{340}$ to LDH units for each condition (Wroblewski & LaDue, 1955).

### *Immunoblot analysis*

Immunoblotting was performed as previously described (Militante *et al*., 1997). Samples of cytosol were electrophoretically separated through 8% polyacrylamide gels containing 0.1% sodium dodecyl sulphate, electrotransferred to polyvinylidene difluoride membranes in transfer buffer, and blocked for 1 h at room temperature in phosphate buffered saline containing 0.05% Tween 20 (PBST) and 1% bovine serum albumin. Membranes were then incubated overnight at 4°C with anti-iNOS monoclonal antibody (1 : 1500 dilution). After washing the membranes and incubating at room temperature with horseradish peroxidase conjugated anti-mouse polyclonal antibody (1 : 3000 dilution), bands were visualized using chemiluminescence and exposure to X-ray film. After chemiluminescence visualization, the membranes were stained with 'India' ink overnight for comparison of protein loading per lane (Harlow & Lane, 1988). Band densities on X-ray films and stained membranes were quantified using a Millipore Bio-Imager. Blots with significant differences between protein loading per lanes were not included in the analysis.

### *Data analysis*

Prism™ software (version 2.01, GraphPad Inc.) was used to perform data transformations and plotting, linear and nonlinear regression, and statistical analyses. For plotting purposes and statistical analyses, the pooled sample from ($n$) total experiments were used as the data set.

To include the variance within control groups in the nonlinear regression analysis, the alcohol concentration for the control group (0 M) was set equal to $10^{-5}$ M prior to logarithmic transformation of concentration. The nonlinear regression variables for maximum and minimum effects were set as constants at 100 and 0% prior to curve-fitting to obtain best-fit estimates for the slope of the curve (Hill coefficient; $n_H$) and effective concentration for 50% effect ($IC_{50}$ or $EC_{50}$). Because $IC_{50}$ values are log-normal distributed (Hancock *et al*., 1988), they are presented with their 95% confidence intervals (CI). In the case where total cell protein was used as the dependent variable, the model-generated $IC_{50}$ and CI values are presented despite the fact that several short chain alkanols did not cause a 50% reduction in total cell protein even at the highest concentration tested. Correlation analyses on data collected from cultures treatment with the maximum tested concentration of the same alkanol used nonweighted linear regression between independent measurements, after normalization to their respective control values.

### *Drugs and materials*

Rat C6 glioma cells (CCL107) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Dulbecco's modified Eagle's medium with high glucose was from MediaTech (Gaithersburg, MD, U.S.A.) and characterized foetal bovine serum was from Hyclone (Logan, UT, U.S.A.). Phorbol 12-myristate 13-acetate, *Salmonella typhimurium* lipopolysaccharide, the lactic acid dehydrogenase diagnostic kit, NADPH, FAD, protease inhibitors, N-(1-naphthyl)ethylenediamine, sulphanilamide, Tris, HEPES, bovine serum albumin, sodium nitrite, 1-pentanol, 1-heptanol, 1-octanol and 1-decanol were all obtained from Sigma (St. Louis, MO, U.S.A.). 5,6,7,8-tetrahydro-L-biopterin dihydrochloride was from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Methanol and 1-propanol were from J.T. Baker Inc. (Phillipsburg, NJ, U.S.A.), 95% ethanol was obtained from Aaper Alcohol and Chemical Co. (Shelbyville, KY, U.S.A.). SuperSignal CL-HRP Substrate System and BCA Protein Assay Reagent A were from Pierce (Rockford, IL, U.S.A.). Horseradish peroxidase conjugated anti-mouse polyclonal antibody and [$^3$H]-L-arginine (specific activity = 60 Ci mmol$^{-1}$) were obtained from Amersham Life Science (Arlington Heights, IL, U.S.A.). Acrylamide, TEMED, L-glycine, and sodium dodecyl sulphate were from BioRad (Richmond, CA, U.S.A.). Clone 6 anti-iNOS monoclonal antibody was obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Brilliant black ink was from Rotring GmbH (Hamburg, Germany).

## Results

### *Inhibition of intact cell activity*

Alkanols of $\leqslant 7$ carbon chain length inhibited 24 h nitrite accumulation in culture medium of C6 cells induced with PMA plus LPS (Figure 1). The potency of inhibition increased with increasing carbon chain length, up to and including 1-heptanol (Table 1). Recovery of total cell protein was also reduced in a concentration-dependent manner by alkanols, with potency increasing with carbon chain length (Table 1). Alkanols C-1 to C-5 were considerably less potent at reducing total cell protein, such that $>50\%$ reductions in nitrite accumulations occur with no reductions in total cell protein (results not shown). 1-Heptanol was about half as potent at reducing total cell protein as inhibiting nitrite accumulation. Nevertheless, 2 mM C-7 inhibited nitrite accumulation $>60\%$ with only a 6% reduction in total cell protein (results not shown). Inhibition of nitrite accumulation by increasing alkanol concentration appeared cooperative, as indicated by $n_H$ values significantly greater than 1.0, except in the case of 1-pentanol (Table 1).

The effect of longer chain alkanols (C-8 and C-10) was examined up to their saturation limits. These alkanols were found to be equipotent for reducing total cell protein and inhibiting nitrite accumulation (Table 1). Inhibition of nitrite accumulation was never observed without an equivalent reduction in total cell protein (results not shown), indicating the reduction in nitrite accumulation was due to cytotoxicity.

Linear regression analysis indicated no significant differences in the slope of the lines relating percentage control nitrite vs concentration or percentage control protein vs concentration, for either alkanol ($P=0.682$ for C-8 and $P=0.907$ for C-10). In contrast, these regression lines were very significantly different when the same comparisons were made for alkanols C-1 to C-7 (results not shown).

We investigated several possibilities underlying the inhibitory effect of short chain alkanols on nitrite accumulation observed with intact cells. These included direct interference with the assay for nitrite, direct effects on iNOS enzyme function, nonspecific effects due to cytotoxicity, and effects on iNOS protein expression.

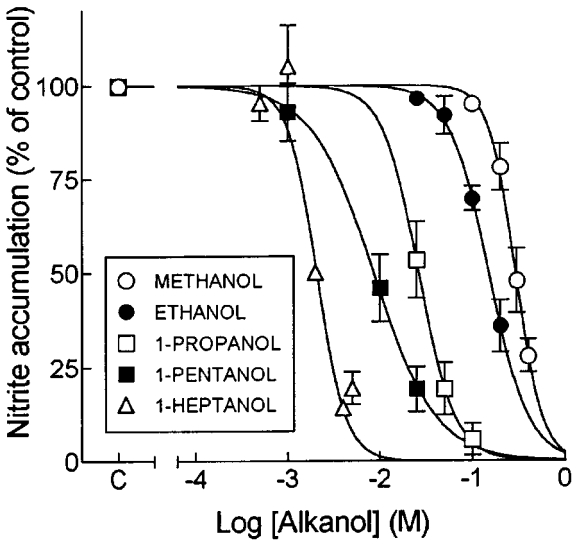


**Figure 1** Alkanol inhibition of 24 h nitrite accumulation by C6 glioma cells exposed simultaneously to 400 ng ml$^{-1}$ PMA plus 500 ng ml$^{-1}$ LPS and the indicated concentration of alkanol. C = control cells without alkanol addition. Sample sizes are: $n=5$, (C-1, methanol); $n=3$, (C-2, ethanol); $n=3$, (C-3, 1-propanol); $n=6$, (C-5, 1-pentanol); $n=6$, (C-7, 1-heptanol). Individual samples are the mean of triplicate or quadruplicate dishes. Data presented as mean per cent control ± s.e. Mean control activities (nmole nitrite produced per mg protein) are: C-1, 15.6 ± 6.4; C-2, 10.5 ± 2.2; C-3, 14.2 ± 6.0; C-5, 7.6 ± 2.1; C-7, 15.7 ± 2.5. See Table 1 for $IC_{50}$ values and Hill slopes.

### *Direct interference with the nitrite assay*

For these experiments, C-1 to C-7 alkanols were analysed at their highest respective concentrations used for intact cell exposures (see Methods). They were dissolved in serum-free medium and used to prepare a nitrite standard curve. Addition of an alkanol, as compared to no addition, was found to slightly, though significantly ($P<0.0001$) enhance colour development upon reaction of equal amounts of nitrite with the Griess reagent (results not shown). Depending on the alkanol, the change in slope resulted in a 3–9% decrease in the estimated nitrite content at a fixed absorption value when compared to the estimated nitrite content made at the same absorbance in serum-free medium with the alkanol. However, because the nitrite content of alkanol-containing medium was routinely determined by comparison to a standard curve prepared without any alkanol, the net effect was a slight overestimation of nitrite content for samples from cells exposed to the alkanol.

### *Direct effects on iNOS enzyme activity*

A cytosolic fraction isolated from induced control C6 cells was used to measure direct effects of alkanols on iNOS enzyme activity. The *in vitro* conversion of [$^3$H]-L-arginine to [$^3$H]-L-citrulline was compared in the absence or presence of alkanols at the highest concentration used on intact cells (Figure 2). One-way ANOVA indicated a significant difference ($P=0.0037$), due to a significant reduction in activity in the presence of 25 mM 1-pentanol compared to control activity ($P<0.01$). No other alkanols caused significant differences.

To determine if the alkanols have *in situ* actions that may affect nitrite production, we tested the series of alkanols for post-induction effects on nitrite accumulation. Alkanols were present at their highest respective test concentrations during the 24–48 h interval following initiation of induction at time = 0 h (Figure 3). No effects were observed with exposure to 400 mM methanol and 200 mM ethanol, but 1-propanol (100 mM), 1-pentanol (25 mM) and 1-heptanol (5 mM) each significantly decreased post-induction nitrite accumulation by about 25% relative to control ($P<0.05$). The lack of effect by 200 mM ethanol is consistent with previous results showing no post-induction effect at 100 mM ethanol (Militante *et al.*, 1997).

**Table 1** Fitted parameters for inhibition of iNOS activity and reduced total cell protein

| *Alkanol* | *iNOS* $IC_{50}$ (nM) [95% CI] | *Protein/dish* $IC_{50}$ (nM) [95% CI] | *iNOS* $n_H$ (± s.e.) | *Protein/dish* $n_H$ (± s.e.) |
|---|---|---|---|---|
| Methanol | 294 | 74,370 | 3.03 ± 0.14 | 0.45 ± 0.03 |
| $n=5$ | [281–307] | [23,210–238,300] | | |
| Ethanol | 151 | 775 | 2.05 ± 0.08 | 1.75 ± 0.20 |
| $n=3$ | [143–160] | [466–1,288] | | |
| 1-Propanol | 26.6 | 310 | 2.22 ± 0.06 | 1.35 ± 0.12 |
| $n=3$ | [25.4–27.7] | [188–511] | | |
| 1-Pentanol | 8.59 | 80.5 | 1.29 ± 0.07 | 1.07 ± 0.03 |
| $n=6$ | [7.34–10.05] | [33.2–195.1] | | |
| 1-Heptanol | 2.07 | 5.61 | 2.78 ± 0.55 | 2.05 ± 0.77 |
| $n=6$ | [1.73–2.48] | [3.57–8.82] | | |
| 1-Octanol | 2.04 | 2.2 | 1.99 ± 1.99 | 1.39 ± 0.67 |
| $n=5$ | [0.22–19.23] | [0.51–9.57] | | |
| 1-Decanol | 0.48 | 0.52 | 3.45 ± 1.65 | 5.22 ± 0.45 |
| $n=4$ | [0.33–0.73] | [0.49–0.56] | | |

Mean $IC_{50}$ values and their 95% confidence intervals (CI), and mean Hill coefficients with their standard error of the means (s.e.) were determined by curve fitting using nonlinear regression. Squares of the correlation coefficients ($r^2$) for goodness of fit were >0.9540, except for iNOS inhibition by 1-Octanol ($r^2=0.9315$).

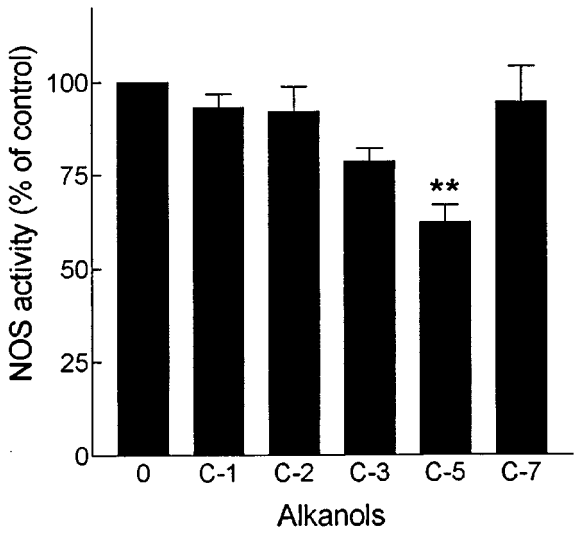


**Figure 2** *In vitro* effect of alkanols on calcium-independent cytosolic NOS activity from PMA plus LPS-induced C6 glioma cells. 0 - no addition ($n=6$); C-1 - 400 mM methanol ($n=5$); C-2 - 200 mM ethanol ($n=3$); C-3 - 100 mM 1-propanol ($n=3$); C-5 - 25 mM 1-pentanol ($n=3$); C-7 - 5 mM 1-heptanol ($n=5$). Each individual sample assayed in triplicate. Data presented as mean per cent control $\pm$ s.e. Mean control (0) activity $= 1.4 \pm 0.2$ nmole citrulline produced per mg protein. One-way ANOVA indicated a significant difference overall ($P=0.0037$). ***Post-hoc* Dunnett's Multiple Comparison Test indicated a significant difference between 0 and C-5 ($P<0.01$).

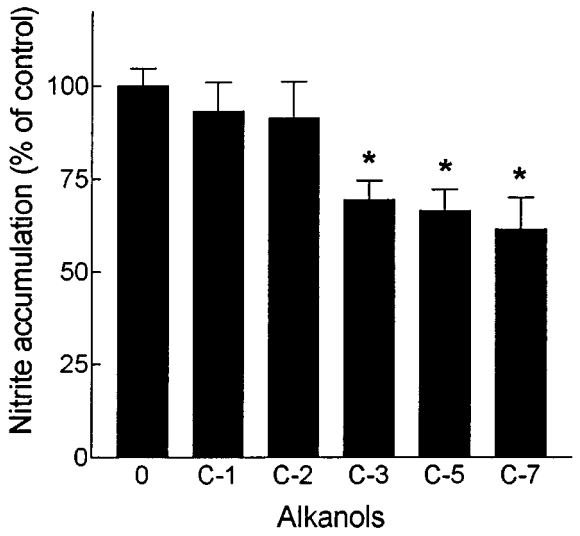


**Figure 3** Effect of alkanol exposure from 24–48 h on post-induction nitrite accumulation of C6 glioma cells induced with LPS plus interferon-$\gamma$ from 0–24 h. Alkanol concentrations are the same as in Figure 3. Data are mean per cent control $\pm$ s.e. ($n=3$) with each experiment performed in quadruplicate. Mean control activity $= 15.9 \pm 2.4$ nmole nitrite produced per mg protein. One-way ANOVA indicated a significant difference between means ($P=0.0004$). **Post-hoc* Dunnett's Multiple Comparison Test indicated significant differences between 0 and C-3, C-5, and C-7 ($P<0.05$).

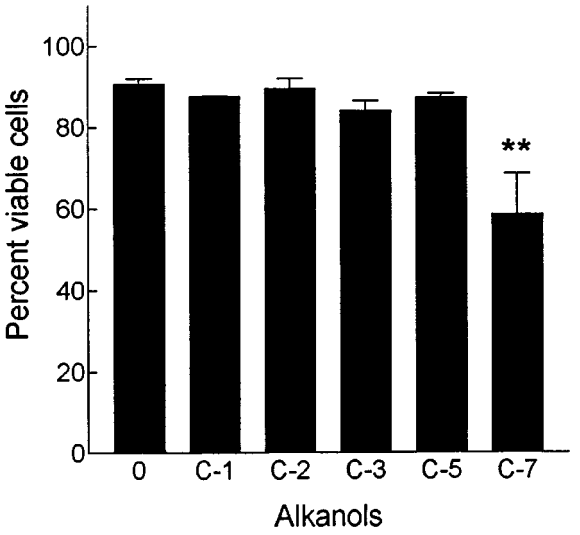


**Figure 4** Effect of 24 h alkanol exposure on the viability of C6 glioma cells stimulated with PMA plus LPS measured by the trypan blue exclusion test. Alkanol concentrations are the same as in Figure 3. Values presented as mean per cent viability $\pm$ s.e. ($n=2-6$), with each individual experiment performed in quadruplicate. Control (0) cell counts are 915 dead cells per 9,863 total cells. One-way ANOVA indicated significant differences between group means ($P=0.0014$). ***Post-hoc* Dunnett's Multiple Comparison Test indicated a significant difference between 0 and C-7 ($P<0.01$).

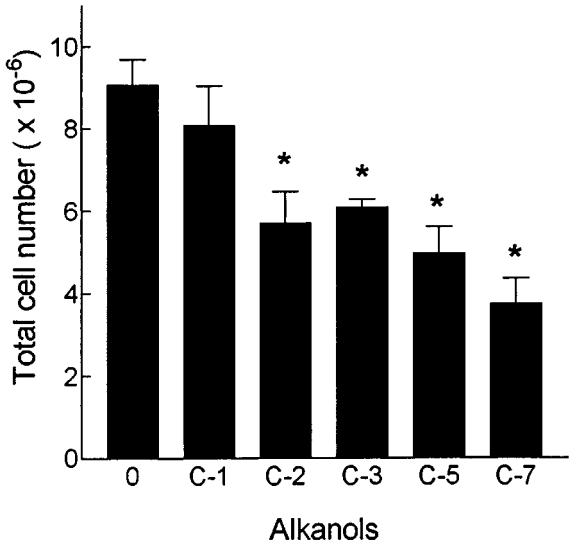


**Figure 5** Effect of 24 h alkanol exposure during PMA plus LPS induction on number of C6 glioma cells recovered from the culture dish. Values are mean cell number $\pm$ s.e. ($n=2-6$), with each individual experiment performed in quadruplicate. Alkanol concentrations are the same as in Figure 3. One-way ANOVA indicated significant differences between group means ($P<0.0001$). **Post-hoc* Dunnett's Multiple Comparison Test indicated significant differences between 0 and C-2, C-3, C-5, and C-7 ($P<0.05$).

### *Cytotoxic effects of alkanols on C6 glioma cells*

Two measures other than recovery of total cell protein (see above) were used to gauge the degree of cytotoxicity resulting from a 24 h exposure of intact cells to alkanols at their highest respective concentrations tested. The first was the trypan blue exclusion test, where the per cent viability is computed based on the number of cells that stain blue. The results are shown in Figure 4. A two-way ANOVA indicated no main effect of the presence or absence of induction on the results ($P=0.47$), so the data were collapsed and a one-way ANOVA was used to test for significant effects of alkanols on viability. The analysis indicated a significant difference overall ($P=0.0014$) and Dunnett's multiple comparison test showed a significant

decrease in per cent viability only for cells exposed to 5 mM 1-heptanol ($P<0.01$).

Analysis of the total number of cells recovered on the dishes after alkanol exposure confirmed what was previously observed using total cell protein measurements; high concentrations of alkanols with two or more carbons significantly decreased the number of cells recovered from the culture dishes (Figure 5).

Release of the intracellular enzyme LDH into the culture medium during exposure of C6 cells to alkanols was used as a second indicator of cytotoxicity. Because of the presence of endogenous pyruvate in the Dulbecco's modified Eagle's medium, or a less likely possibility of the release of other NADH-reducing enzymes from the cells, a time-dependent decline in the absorbance at 340 nm was observed in the absence of added pyruvate. Therefore, each media sample was assayed in both the absence and presence of exogenous pyruvate to determine LDH activity. There appeared to be a tendency for LDH release to increase with carbon chain length (Figure 6), but the release in the presence of 400 mM methanol and 200 mM ethanol was not statistically different from control. Exposure to 100 mM 1-propanol caused significantly greater release than control ($P<0.01$), while LDH activity measured following exposure to 25 mM 1-pentanol was significantly different than control and all other alkanol groups ($P<0.01$). In contrast to all other groups, LDH activity decreased upon addition of pyruvate (see Methods) to media collected from cells exposure to 5 mM 1-heptanol (Figure 6).

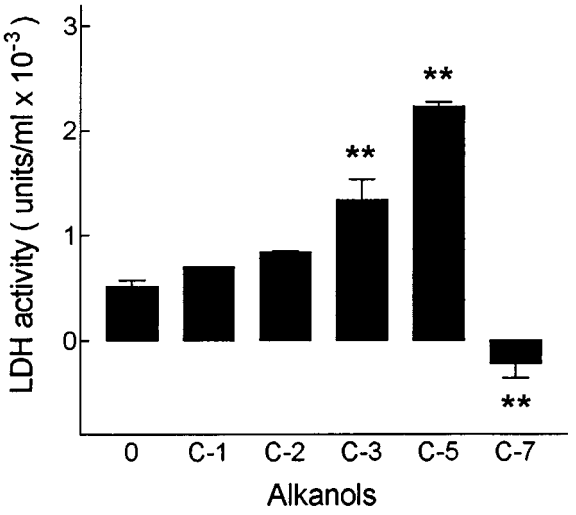


**Figure 6** Effect of 24 h alkanol exposure on the release of lactic acid dehydrogenase into the culture medium of C6 glioma cells stimulated with PMA plus LPS. Values are mean units ml$^{-1}$ ± s.e. ($n=2$ or 4) for pyruvate-stimulated activity. Each individual experiment performed in triplicate. One-way ANOVA indicated a significant difference between groups ($P<0.0001$). ****Post-hoc*** Dunnett's Multiple Comparison Test indicated significant differences between 0 and C-3, C-5, and C-7 ($P<0.01$).

### *Correlation analysis of experimental data*

To determine if the decrease in nitrite accumulation observed during exposure of intact cells to alkanols can be accounted for by actions on the functional enzyme or by cytotoxicity, a linear regression analysis was performed (Table 2). Results indicate that the per cent decrease in nitrite accumulation of intact cells during exposure to C-1 to C-7 alkanols at their maximum tested concentrations does not correlate with any direct action on the iNOS enzyme, nor with effects related to cytotoxicity. As expected, a significant correlation was observed between percentage total cell protein per dish and percentage total cell number per dish ($P=0.022$). Other significant correlations between experimental data were observed also (Table 2), probably reflecting a general tendency for C-5 and C-7 alkanols to cause decreases in the experimental parameter under investigation.

In contrast to the data in Table 2, highly significant correlations were obtained between the logarithm of the $IC_{50}$ value and their 95% confidence interval for C-1 to C-7 alkanols and their carbon chain length ($P=0.0023$, Figure 7A), the logarithm of their membrane:water partition coefficient ($P=0.0014$, Figure 7B), and their molar volumes ($P=0.0023$, results not shown). Values for the latter two measurements were taken from published data (McCreery & Hunt, 1978).

### *Reductions in iNOS immunoreactivity*

To determine the effects of short chain alkanols on the amount of iNOS protein expressed after 24 h exposure, immunoblots were prepared on cytosol fraction from control cells and cells exposed to 150 mM ethanol, 10 mM 1-pentanol, and 2 mM 1-heptanol (Figure 8A). The densitometry results from four independent experiments indicate a statistically significant reduction in iNOS protein content following short chain alkanol exposure (Figure 8B). Furthermore, the degree of reduction in iNOS protein content was positively correlated

**Table 2** Correlation analysis for several measures of direct and indirect effects on iNOS activity and cell viability

| | *% Nitrite accumulation* | *% In vitro inhibition* | *% Inhibition post-induction* | *% Viability* | *% Total cell number/dish* | *% LDH release* | *% Total cell protein/dish* |
|---|---|---|---|---|---|---|---|
| % Inhibition of nitrite accumulation using intact cells | | $r^2=0.3207$ | $r^2=0.5640$ | $r^2=0.1385$ | $r^2=0.5011$ | $r^2=0.0835$ | $r^2=0.4531$ |
| % *In vitro* inhibition | | | $r^2=0.3660$ | $r^2=0.0251$ | $r^2=0.1887$ | **$r^2=0.8000$** **$P=0.0161$** | $r^2=0.1041$ |
| % Post-induction inhibition | | | | $r^2=0.4316$ | **$r^2=0.7165$** **$P=0.0336$** | $r^2=0.0406$ | **$r^2=0.7927$** **$P=0.0174$** |
| % Viability | | | | | $r^2=0.4488$ | $r^2=0.3397$ | **$r^2=0.7631$** **$P=0.0230$** |
| % Decrease in cell number/dish | | | | | | $r^2=0.0023$ | **$r^2=0.7671$** **$P=0.0222$** |
| % LDH release | | | | | | | $r^2=0.0174$ |

The data are presented as the square of the correlation coefficient ($r^2$) determined from linear regression analysis. A statistically significant correlation with the probability is bolded.

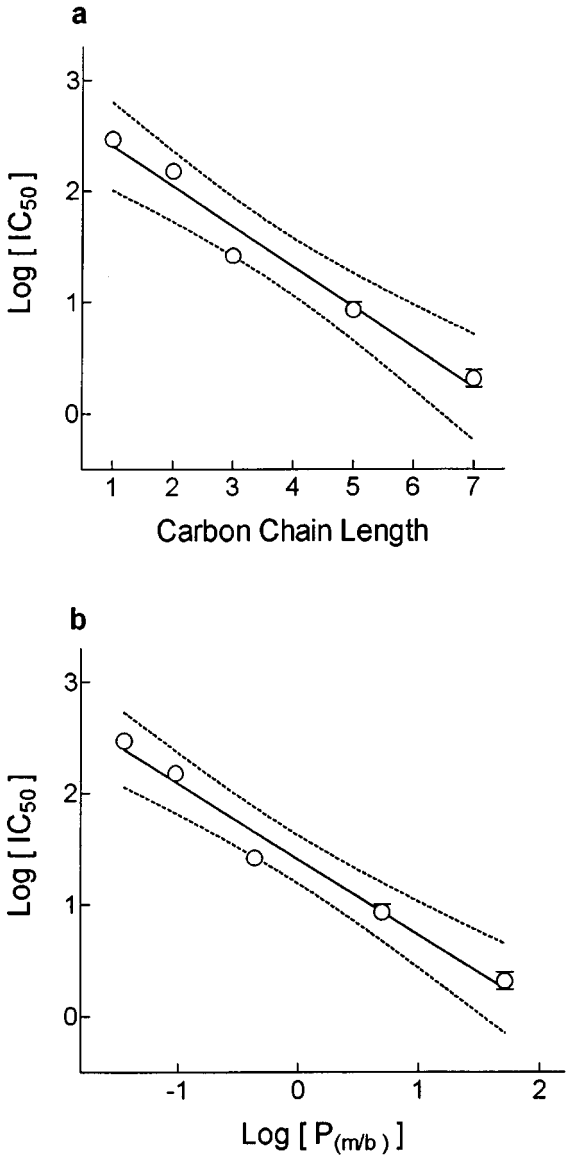


**Figure 7** Correlation between Log ($IC_{50}$), with 95% confidence intervals and (a) carbon chain length, or (b) Log ($P_{m/b}$) for C-1 to C-7 alkanols. $IC_{50}$ and 95% confidence interval values are from Table 1. Values for the membrane/buffer partition coefficient ($P_{m/b}$) are taken from McCreery & Hunt, 1978. Linear regression analysis indicated highly significant correlations for both measures; (a) $r^2=0.9692$, $P=0.0023$ and (b) $r^2=0.9777$, $P=0.0014$.

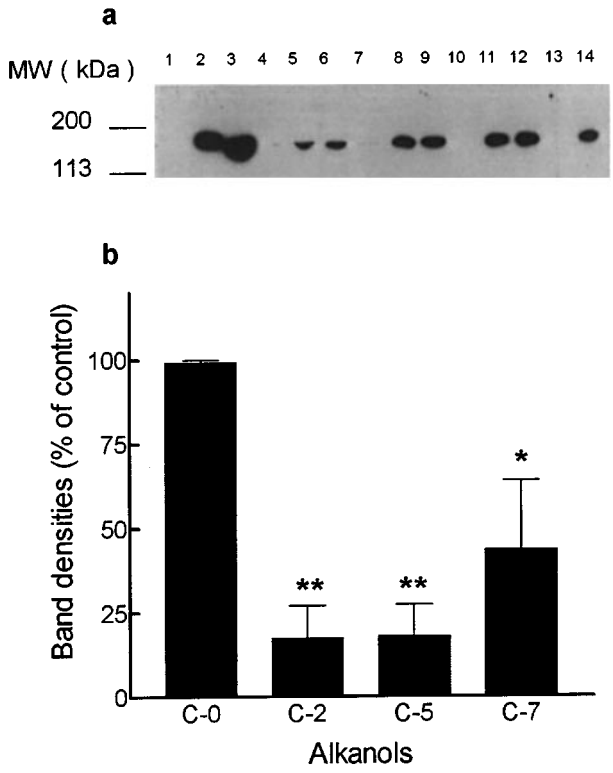


**Figure 8** Reductions in PMA plus LPS-induced iNOS protein immunoreactivity of C6 glioma cells by 24 h exposure to alkanols. (a) Representative immunoblot with location of molecular weight markers at left. Lanes 1, 4, 7, and 10 are cytosolic extracts from unstimulated cells exposed to no alkanol, 150 mM ethanol, 10 mM 1-pentanol and 2 mM 1-heptanol, respectively. Lanes 2, 3, 5, 6, 8, 9, 11, and 12 are independent extracts from sister cells stimulated with PMA plus LPS in the presence of no alkanol, 150 mM ethanol, 10 mM 1-pentanol and 2 mM 1-heptanol, respectively. Lane 13 is blank and lane 14 is an iNOS protein positive control. Equal amounts of protein were loaded in lanes 1–12. No significant differences were observed for an abundant protein band stained with 'India' ink. Mean (±s.d.) relative intensities are: C-0, 1155±445; C-2, 1470±297; C-5, 945±148; C-7, 945±148 ($F=1.489$, $P=0.3453$ by one-way ANOVA). (b) Results of densitometric analysis on four independent immunoblots, each containing two independent extracts from sister cultures, from four independent experiments. Data are mean per cent control±s.e. 0=no alkanol treatment, C-2=150 mM ethanol, C-5=10 mM 1-pentanol, and C-7=2 mM 1-heptanol. *,**Post-hoc Dunnett's Multiple Comparison Test indicated significant differences between 0 and C-2 ($P<0.01$), 0 and C-5 ($P<0.01$), and 0 and C-7 ($P<0.05$).

with the degree of reduction in nitrite accumulation for these four experiments (slope=1.36, $r^2=0.9964$, $P=0.0018$).

## Discussion

### *Does alkanol inhibition of iNOS exhibit a cutoff phenomenon?*

The acute inhibitory effect of ethanol and other intoxicating alcohols on brain function has been interpreted previously in terms of alcohols partitioning into the lipid phase of the cell (Seeman, 1972; Goldstein, 1984). This interpretation stems, in part, from the high correlation between behavioural measures of alcohol potency and lipophilicity (McCreery & Hunt, 1978) or membrane disordering (Lyon *et al*., 1981) measures, which coincidentally exhibits cutoff effects beyond a certain carbon chain length. However, several studies in recent years have shown that alkanols, secondary alcohols and anaesthetics can have effects on purified protein systems that also correlate well with measures of lipophilicity and exhibit cutoff effects, (Wood *et al*., 1991; Machu & Harris, 1994; Li *et al*., 1994; Franks & Lieb, 1985) suggesting a hydrophobic pocket on a protein as a site of action. Interestingly, the point of cutoff appears unique to each system, suggesting these compounds act at several different hydrophobic pockets.

The present results do not provide clear-cut evidence for a cutoff phenomenon for acute alkanol inhibition of iNOS activity, but the data are very suggestive. For example, only alkanols with $\leqslant 7$ carbons were observed to significantly reduce nitrite accumulation at concentrations that did not also cause an equivalent reduction in total cell protein. Also, the computed $IC_{50}$ values for inhibition of nitrite accumulation by the C-8 and C-10 alkanols were 2–4 times less than their predicted values based on the regressions shown in Figure 7. The answer to whether alkanol-reduced nitrite accumulation shows a true cutoff phenomenon must await development of

cell-free systems with which to study this effect, so that cytotoxicity does not compromise interpretation of results.

### *What causes reduced nitrite accumulation?*

Several possibilities were explored to better understand how 24 h exposure to C-1 through C-7 alkanols caused a concentration-dependent reduction in nitrite accumulation in cultures of C6 glioma cells induced with PMA plus LPS. The approach taken was to measure other possible side effects of alkanols at their maximum tested concentrations that might reduce nitrite measurement of iNOS activity. The data, converted to per cent of control activity, was then subjected to correlational analyses to determine if any alkanol effects and the corresponding per cent inhibition of nitrite accumulation for the same alkanol might be related.

The results indicate that the reduction in nitrite accumulation cannot be accounted for by any direct interference of the alkanols with the nitrite assay, although there is a small enhancement in colour development by the different alkanols. Similarly, the reduction in nitrite accumulation by different alkanols cannot be explained by a direct effect on the functional iNOS enzyme because all the alkanols except 1-pentanol failed to significantly inhibit *in vitro* activity of the expressed iNOS enzyme. The reduction in nitrite accumulation in the media of intact C6 glioma cells exposed to 25 mM 1-pentanol, however, may be due in part to some *in situ* inhibition of the iNOS enzyme. This is supported by the finding that exposure of intact C6 cells already expressing the iNOS enzyme to 1-pentanol caused a significant reduction in subsequent nitrite accumulation.

A reduction in post-induction nitrite accumulation was also observed with 1-propanol and 1-heptanol, but not with methanol or ethanol. However, none of these alkanols directly inhibited *in vitro* iNOS activity. These results suggest that *in situ* inhibition of the iNOS enzyme, as well as possible alterations in substrate or co-factor availability, may contribute to the reduced nitrite accumulation observed using intact cells exposed to 1-propanol, 1-pentanol and 1-heptanol. *In situ* inhibition of the iNOS enzyme cannot, however, account for the majority of the nitrite inhibition by these alkanols or for any of the nitrite inhibition by methanol and ethanol.

Loss of cell viability was also eliminated as an underlying mechanism for the reduced nitrite accumulation. Alkanols with five or less carbons failed to reduce the exclusion of trypan blue by C6 glioma cells exposed to maximum tested concentrations, while at the same time producing 64–94% inhibition of nitrite accumulation. General cytotoxic effects, measured as LDH release, also cannot adequately explain the observed reductions in nitrite accumulation using intact cells, since C-1, C-2 and C-7 alkanols were not found to be cytotoxic. However, the cytotoxicity observed for 1-propanol and 1-pentanol possibly contributed to their overall reduction in nitrite accumulation. Effects due to hypertonicity can be eliminated, as well, since significant reductions were observed with C-5 and C-7 alkanol concentrations of 2–10 mM.

It has been suggested that ethanol and other aliphatic alcohols might interact chemically with nitric oxide to form nitrosylated compounds such as ethyl nitrite, thus sequestering the nitric oxide and explaining their ability to inhibit nitric oxide-mediated relaxation of rat anococcygeus muscle and gastric fundus (Rand & Li, 1994). The possible nitrosylation of alkanols by nitric oxide seems to be of little significance for two reasons. First, as already discussed, the accumulation of nitrite, a stable oxidation product produced in direct proportion to nitric oxide, was not reduced when cells already expressing iNOS and producing nitric oxide were exposed to 400 mM methanol or 200 mM ethanol for 24 h. In addition, although 100 mM 1-propanol, 25 mM 1-pentanol, and 5 mM 1-heptanol each reduced nitrite accumulation in the media of cells already expressing iNOS, the degree of reduction was equivalent for the three alkanols, about 25%, despite a 20 fold difference in concentrations between the different alkanols. Second, studies on the interaction of 100 mM ethanol with the ferrous-nitrosyl complex of Soybean lipoxygenase-1 found no evidence of ethanol interacting chemically with nitric oxide, and instead observed that ethanol blocked the *p*H-sensitive shift in the ferrous-nitrosyl EPR spectrum, probably through hydrogen bonding between ethanol and the non-haeme iron (Nelson, 1987).

### *How do alkanols reduce nitrite production?*

One property that correlates highly with the potency of C-1 to C-7 alkanols to inhibit nitrite accumulation by intact cells is lipophilicity. These results are consistent with inhibition of iNOS induction due to alkanol interaction with a hydrophobic site that can accommodate primary alcohols of seven carbons or less. Given what we currently know about such a correlation, it is not possible to conclude whether this indicates an action on membrane lipids or binding to a hydrophobic pocket of a protein involved in transducing the induction signal to the nucleus. What is clear, however, is that the inhibition by short chain alkanols is not a direct effect on iNOS enzyme activity or a consequence of cytotoxicity.

It was suggested previously that the ethanol-induced reduction in nitrite accumulation seen with C6 cells resulted from decreased iNOS expression (Syapin, 1995). This suggestion is now supported by direct evidence that incubation with ethanol reduces steady-state levels of both iNOS protein and iNOS mRNA (Militante *et al*., 1997). The present findings, including decreased steady-state levels of specific iNOS protein immunoreactivity, suggests that other short chain alkanols act the same way. Thus, identification of the site of action for short chain alkanol inhibition of iNOS expression will provide important new information on molecular targets for ethanol-altered gene expression.

Inhibition of nitrite accumulation during iNOS enzyme induction was found to be a property shared by short chain primary alcohols. Several explanations were sought for the decrease in nitrite accumulation, including possible direct effects on iNOS catalytic activity and indirect effects due to cytotoxicity. Only inhibition by longer chain alkanols ($\geqslant$C-8) is due to cytotoxicity. Therefore, the results support a specific interaction of short chain alkanols with an important intermediary step in the iNOS induction pathway, thus confirming and extending previous studies with ethanol.

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